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# Regenerative Secretoma of Adipose-Derived Stem Cells from Ischemic Patients

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## Abstract

Patients with terminal ischemia often reveal chronic limb and foot ulcers with subsequent risk of infection and/or amputation. Adipose-derived Stem Cells (ASC) may secrete angiogenic and regenerative factors. The autologous transplantation of such cells is considered to be an attractive therapeutic strategy, but their functional properties of ASC are influenced by many biochemical and biophysical stimuli of the microenvironment. Thus, patient-derived ASC might not be functionally competent.

To study ASCs in ischemic disease, we have generated ASC lines from fat tissue of twelve ischemic patients. Lines were characterized for cell surface phenotype, multipotent capacities, and production of factors involved in wound healing. We succeeded to amplify ASC lines from all twelve patients and confirmed an ASC identity by their ability to: (i) adhere and grow on a plastic surface in standard culture conditions; (ii) express an ASC expression profile; (iii) differentiate in vitro into adipocytes, osteoblasts, and chondroblasts. Full transcriptome analysis of four selected lines showed a gene expression profile compatible with healing properties including all of the functional families involved in the wound healing process: extracellular matrix proteins, cell growth factors, pro-inflammatory cytokines, angiogenic factors, and matrix remodeling proteins.

Our pilot study confirms that high-quality adipose stem cells can be easily derived from ischemic patients. Their transcriptome and secretome show a regenerative profile which makes them promising candidates for autologous therapy of chronic ulcers.

**Keywords:** Chronic ulcers; Chronic ischemia; Adipose stem cells; Neovascularization; Diabetic foot infection; Secretoma; Proof-of-principle; Laboratory

## Introduction

An important number of elderly and/or diabetic patients have advanced chronic ischemic limb disease with severe and chronic ulcers. In the absence of correct wound healing, these ulcers become a risk of permanent pain, infection, and amputation. Vascular surgery or angioplasties are not always feasible, especially with a long-standing evolution and microangiopathy. Thus, alternative strategies other than mechanical neo-vascularization are needed. Most of the prior innovative studies used angiogenic growth factors alone and reported a limited efficacy [1]. This prompted many author groups to investigate cell-based therapies. The local injection of unselected bone marrow or peripheral blood-derived mononuclear cells was encouraging, but often not sufficiently efficacious for complete revascularization; probably due to the insufficient delivery of regenerative factors from a too complex mixture of cell populations [2]. Other researchers investigated the autologous bone marrow cell transplantation to increase limb perfusion, with low success [3]. Mesenchymal stem cells of adult fat tissues, called Adipose-derived Stem Cells (ASC), are particularly attractive for the local treatment of ischemic ulcers due to their easy accessibility with minimal invasiveness and ethical concerns, and their ability to produce most of the healing/angiogenic factors [4]. However, the proof of feasibility in ischemic patients is almost lacking.

There is a general consensus about the potential for clinical treatment of ischemic ulcers [5]. For ASC isolation, superficial

subcutaneous fat is usually harvested by resection or lipoaspiration. The viability, yield and growth characteristics of ASC are influenced by the type of harvesting procedure, as well as by many biochemical and biophysical stimuli in the in vivo microenvironment, such as fluid shear stress, hydrostatic pressure, and produced factors. Mesenchymal stem cells isolated from diabetic patients yielded dysfunctions in oxidative stress, autophagy healing properties in mice [6] and VEGF expression [7]. However, this observation is controversial and reports from diabetic rats [6] or patients [7] still retain the ability to promote wound healing. We thus describe a proof-of-principle pilot study regarding the feasibility of amplifying ASC from patients with long-standing ischemia and/or diabetes mellitus from various origins for a theoretical future autologous use for chronic ulcer regeneration.

## Materials and Methods

### Patients and definitions

From June 2015 to April 2018, we intraoperatively sampled visually healthy fat tissue pieces from the abdomen, limb or feet of ischemic patients hospitalized for any orthopedic or plastic surgery. The tissues were not infected, nor sampled from necrotic areas. The surgeons introduced a hollow blunt-tipped cannula into the subcutaneous space through small (1 cm) incisions or cut the pieces off. The Ethical Committee of Geneva approved the study (NAC 14-183). We defined chronic limb and foot ischemia on topical signs and symptoms.

### ASC isolation and culture

We separated fat from the skin with scalpels, fragmented it after the surgery in small pieces of 2 mm length, and immediately immersed it into HBSS medium (1 volume of medium to 1 volume of fat). The HBSS contained calcium and magnesium (Thermofisher), penicillin-streptomycin 1 U/ml (Thermofisher). The suspension was washed twice with 1 volume of HBSS buffer, followed by elimination of the oil layer on the top of the suspension. After washing, the suspension was maintained under constant agitation at 37°C for 1 hour in the presence of collagenase NB4 0.6 U/ml (Amsbio). We then filtered the suspension by cell strainers (100 µm) to remove residual fragments and centrifugated at 1200 x g during 10 minutes. Then we washed the pellet three times HBSS, prior to plating it on tissue culture flasks at a density of 85 000 cells/cm$^2$, using the following media: DMEM 4.5 g/l glucose with Glutamax (Thermofisher), Fetal Calf Serum (FCS, Thermofisher) or fibrinogen-depleted human platelet lysate 10% (Cook), penicillin/streptomycin 1 U/ml (Thermofisher). Cells were passaged by enzymatic dissociation using 0.5% trypsin/0.2% EDTA (Thermofisher) (maximum for ten times).

### Preparation of embryoid bodies

HS420 embryonic stem cell line was cultured on matrigel (corning) -coated tissue culture flasks in nutristem medium (Biological Industries) until 50% of confluency. Then, 10 µM rock inhibitor Y27632 was added in the nutristem culture media. After 24h, cells were detached with Accutase (Thermofisher) and aggregated into spheroids of 1000 cells through by using Aggrewell-400 (STEMCELL technologies). 24h after aggregation, spheroids were cultured for 10 days in differentiation medium in suspension under constant agitation in the differentiation medium. Then, spheroids were plated in matrigel-coated dishes and cultured in adherence for an additional 11 days in differentiation medium. Differentiation medium: KnockOut DMEM (Thermofisher), 15% KnockOut serum replacement (Thermofisher), 2 mM L-glutamine (Sigma-Aldrich), 1% Penicillin/Streptomycin solution (Life Technologies), 1% of MEM Non-Essential Amino Acids Solution (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma Aldrich).

### Culture of fibroblasts

Human foreskin fibroblasts (ATCC CCD-1112SK) were cultured in DMEM culture media (Life Technologies) supplemented with 10% fetal calf serum (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were harvested for experiments at 90% confluence.

### Flow cytometry and differentiation of ASC

We analyzed the ASC phenotype with the human stem cell verification kit (R and D Systems). Briefly, cells were incubated with fluorochrome-labeled antibodies for 30 minutes at 4°C in binding buffer, prior to washing them with PBS and analyzing them using the BD AccuriTM-B6 flow cytometer (BD Biosciences). We differentiated ASC into chondrocytes, adipocytes or osteocytes by using culture additives and procedures according to the functional mesenchymal stem cell verification kit (R and D Systems).

### Immunocytochemistry, cytokines measurements and microarrays

We cultured ASC on glass coverslips prior to fixation with paraformaldehyde 0.5%; for 15 minutes at room temperature. The cells were incubated overnight at 4°C in PBS containing 0.3% Triton X-100 and 0.5% bovine serum-albumin with primary antibodies. We incubated the secondary anti-mouse IgM-Alexa 555 antibodies for one hour at +4°C in the same PBS solution. The cells were again incubated with DAPI 1 µg/ml for 10 minutes, prior to final washing and mounting. Supernatants from ASC cultures were collected after three days. We assessed the cytokines in culture supernatants according to the manufacturer's instructions, by using the human cytokine base kit A (R and D Systems) combined with a magnetic Luminex assay (Bio-plex 200, Biorad) and performed microarrays targeting 21 448 mRNA chips on the extracted RNA (Complete GeneChip® Instrument System, Affymetrix®, www.affymetrix.com). The heatmap was done on RMA normalized data produced with the software TAC4.0.1.36 (Biosystems®) using the R package pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) default parameters. The data was used without transformation.

## Results

### Cell lines

We derived twelve ASC lines from twelve different ischemic adults (called iASC); eight in medium containing Fetal Calf Serum (FCS) (Table 1); and four in medium containing Human Platelet Lysate (HPL) (Table 2). All iASC expressed the surface profile defined by the International Federation for Adipose Therapeutics and Science: with positivity for CD73, CD105, CD90 and negativity for CD45, HLA-DR and CD34 [8] (Tables 1 and 2). In the presence of appropriated media and after two weeks, all lines differentiated on plastic towards adipocytes (Figure 1A) expressing the specific marker FABP4 (Figure 1B). All lines also efficiently differentiated towards osteocytes (Figure

1C) expressing osteocalcin (Figure 1D). Furthermore, the cell compaction in the presence of a differentiation medium generated.

| iASC line | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Age | 92 | 81 | 74 | 87 | 84 | 55 | 57 | 70 |
| Gender | M | F | M | F | M | M | M | M |
| Ischemia | Foot | Limb | Thigh | Limb | Limb | Foot | Calcaneus | Stump |
| Disease | Ischemia | Ischemia | Ischemia | Ischemia | Ischemia, abscess | Ulcer | Ischemia, Hepatitis C | Infection, hypertension |
| Chronic ulcer | Yes | Yes | Yes | Yes | No | Yes | No | Yes |
| Diabetic | No | Yes | No | No | No | No | No | Yes |
| Smoker | No | No | No | No | No | No | Yes | No |
| Derivation | 2015 06 29 | 2015 06 29 | 2015 07 06 | 2015 07 08 | 2015 07 28 | 2015 07 28 | 2015 07 31 | 2015 08 03 |
| Fat collection | Abdomen | Abdomen | Abdomen | Abdomen | Abdomen | Abdomen | Abdomen | Abdomen |
| Phenotype | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Differentiation Adipocytes Chondrocytes Osteocytes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Supplement | FCS | FCS | FCS | FCS | FCS | FCS | FCS | FCS |

**Table 1:** Patients characteristics for the derivation of eight iASC in culture conditions containing FCS.

| iASC line | 9 | 10 | 11 | 12 |
|---|---|---|---|---|
| Age | 50 | 64 | 49 | 72 |
| Gender | F | M | M | M |
| Ischemia | Leg | Leg | Foot | Foot |
| Disease | Radiodermitis, synoviosarcoma | Malformation, Gut cancer, alcohol | Trauma, amputation | Diabetes, chronic ulcer, amputation |
| Chronic ulcer | No | No | No | Yes |
| Diabetic | No | No | No | Yes |
| Smoker | No | No | No | No |
| Derivation | 2018 02 21 | 2018 02 26 | 2018 03 13 | 2018 08 13 |
| Fat collection | Leg | Foot | Foot | Foot |
| Phenotype | Yes | Yes | Yes | Yes |
| Differentiation Adipocytes Chondrocytes Osteocytes | Yes | Yes | Yes | Yes |
| Supplement | HPL | HPL | HPL | HPL |

**Table 2:** Patients characteristics for the derivation of four iASC in culture conditions containing HPL.

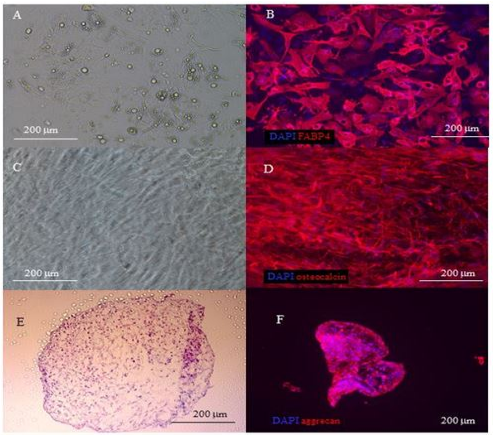


**Figure 1:** Differentiation of iASC 9 into adipocytes, osteocytes, and chondrocytes iASC9 were plated at various cell densities and cultured in two or three dimensions for several weeks in appropriated differentiation media. (A): iASC were plated at the density of 2.1.104 cells/cm$^2$ and differentiated for two weeks in the appropriated medium. After two weeks, adipocytes with oil vacuoles within their cytoplasm were observed; (B): Immunostaining of adipocytes after two weeks of differentiation by using a polyclonal goat anti-mouse FABP4 antibody; (C): iASC were plated at the density of 4.2.103 cells/cm$^2$ and differentiated for two weeks in the appropriated medium. After two weeks, osteocytes were observed; (D): Immunostaining of osteocytes after two weeks of differentiation by using a mouse anti-human osteocalcin antibody; (E): 2.5.105 iASC were pelleted at the bottom of a sterile tube prior to culture three weeks in the appropriated medium. After three weeks, a chondrogenic pellet was formed and cryosectioned for hemalum/eosin coloration; (F): Immunostaining of chondrocytes after three weeks of differentiation by using a goat anti-human aggrecan antibody.

## Gene expression profile of iASC

To estimate the functional secretome for ischemic ulcer regeneration, a gene expression array was performed on the four iASC derived in clinical-grade compatible conditions (iASC9 to iASC12). Hierarchical clustering by using the TAC4.0.1.3.6 software (Applied Biosystems®) showed similarities among all four iASC lines (Figure 2). This suggests a stable and reproducible gene expression profile between

the tested lines. We then analyzed an exhaustive set of positive and negative ASC markers.

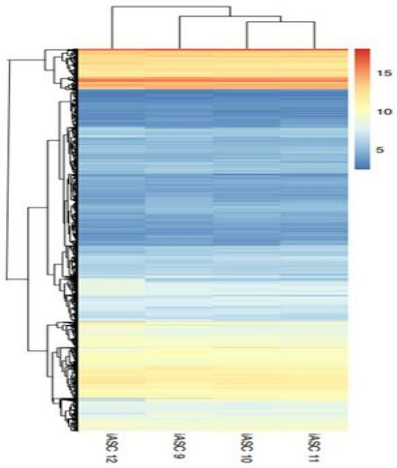


**Figure 2:** Hierarchical clustering of expressed mRNA in 4 iASC. The full transcriptoma of iASC 9-12 was analyzed by GeneChip® Instrument System, Affymetrix®. Hierarchical clustering was performed by the TAC4.0.1.36 applied Biosystem®.

All four lines expressed 14 positive markers and were negative for 10 leucocyte markers, confirming a robust ASC phenotype (Supplementary Figure 1). To estimate the regenerative properties of iASC in terms of possible ischemic ulcer regenerations, we screened in the array the expression of genes from the most important families involved in wound healing: cytokines, chemokines, angiogenic factors, cell growth factors, Matrix-Metallo-Proteinases (MMP), neurotrophic factors. To distinguish the regenerative transcriptome from a possible background of expressed factors in the context of normal cell physiology, controls were added in the analysis.

Embryoid Bodies (EB)-differentiated in vitro from embryonic stem cells were used as "non-regenerative cells" because contained immature cells of the three primitive germ layers representative of most cell types of the body and supposed to not have healing properties. Human Foreskin Fibroblasts (HFF) were used as a "regenerative cell" control because they are actively involved in the healing process and produce a highly regenerative secretome making them also candidates for cell therapy application [9]. Namely, we investigated the pro-inflammatory properties of iASC by a complete analysis of all cytokines. Four cytokines were found to be expressed: IL-6, IL-11, IL-32 to 34 (Figure 3A), but only the pro-inflammatory IL-6 was differentially expressed between EB and iASC/HFF. Screening for chemokines yielded 9 expressed genes (Figure 3A), which are encountered during inflammatory cell recruitment [10-15] and angiogenesis [16]. With the exception of CCL-2, several chemokines were expressed in iASC, but not in HFF and EB. Screening of growth factors revealed several genes expressed (Figure 3B). The family of Insulin Growth Factor Binding Proteins (IGFBP) was well represented with the robust expression of IGF2BP2, IGFBP3, IGFBP4, IGFBP6, and IGFBP7. Additionally, we observed expression of Fibroblast Growth Factors (FGF), including FGF-2, FGF-5, and FGF-7 (or KGF/Keratinocytes Growth factors). Most of them were higher in the four iASC and HFF than EB and showed similar expression between the iASC lines. Screening of angiogenic factors confirmed the presence of 6 genes such as VEGF, ANGPTL-2, ANGPTL-4, and adrenomedullin (Figure 3C). ADM, ANGPTL-2 and VEGF-C display low expression levels in EB and while highly and similarly expressed between the iASC. Quantitatively, the most abundant observed mRNA expression corresponded to matrix-metallo-proteinases (MMP), the key remodeling factors involved in wound healing, with an expression of MMP-1, 2, 3, 9, 14, 16, 19 (Figure 3D). Sometimes higher than EB (MMP-1, 2, 3, 14, 19) and similar between the three iASC, this suggests a substantial role of ASC in subcutaneous tissue remodeling (Figure 4B). Finally, screening of neurotrophic factors was performed and found in all iASC (Figure 3E). Only neurogenin-1 (NGN1) was higher than expressed in EBs, suggesting a potential effect of iASC in nervous system repair.

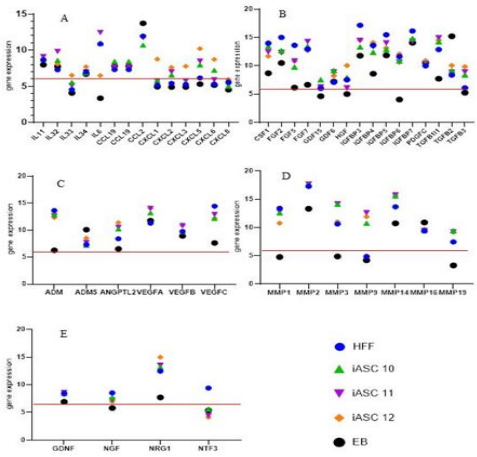


**Figure 3:** mRNA expression of healing factors in iASC, EB, and HFF. The mRNA expression of genes of functional families involved in the healing process was analyzed from the microarray data. A value of 6, on average, for mRNA expression, is considered to be the cutoff of positivity as indicated by the red line. (A): Cytokines and chemokines; (B): Cell growth factors; (C): Angiogenic factors; (D): Matrix-Metallo-Proteinases (MMPs); (E): Neurotrophic factors.

## Healing factors produced by iASC in the culture supernatant

To confirm regenerative properties at the protein level, several cytokines and factors representative of important functional families were measured in the medium from iASC culture by using a highly sensitive Luminex®-based detection method. Measurements were done in equal volumes of media three days after passage 3. Although more variable between iASC lines, the pro-inflammatory cytokines and chemokines IL-1, IL-6, CCL-2 and CXCL-5 were confirmed in all supernatants (Figure 4A-4D); with a predominance of IL-6. The immunosuppressive IL-10, as expected, was not detected (data not shown). IL-8 (CXCL-8) was detected in all supernatants, although at low expression at the mRNA level (data not shown). The potent growth factor FGF-2 was also confirmed in the media (Figure 4E). Likewise, we also detected VEGF (Figure 4F). In contrast to mRNAs, some variability between iASC and biological replicates was noticed; suggesting that external factors at the environmental or post-transcriptional level add some fluctuation into the analyses. Nevertheless, we observed less variability and quantitative

predominance with MMPs; and a similar profile between the four iASC (Figure 5). The quantitative predominance of MMP-2 and MMP-3 secretion was evidenced by large amounts of a factor in culture medium; implying the importance of ASC in tissue remodeling processes.

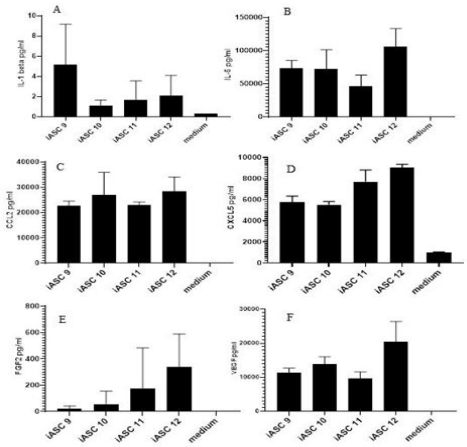


**Figure 4:** Measurement of regenerative factors in supernatants of four ASC lines. iASC were culture until confluency at passage 3. Three days before next confluency, we did not perform medium change before its collection and freezing at -80°C. Factors were measured in each supernatant in biological triplicates by using a sensitive Luminex®-based detection method, targeting IL-1 beta (A): IL-6; (B): CCL2; (C): CXCL5; (D): FGF; (E): VEGF; (F): Results are presenting the average and standard deviation for three biological replicates.

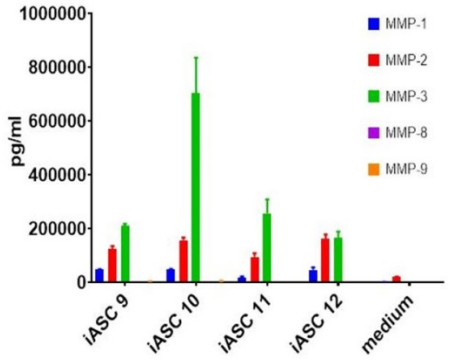


**Figure 5:** Measurement of MMPs in supernatants of four ASC lines. iASC were culture until confluency at passage 3. Three days before next confluency, we did not perform medium change before its collection and freezing at -80°C. MMPs were measured in each supernatant in biological triplicates by using a sensitive Luminex®-based detection method.

## Discussion

In this pilot and proof-of-principle study, we successfully derived ASC lines from the fat of ischemic adults and expand them in vitro in animal-free culture conditions. A more in-depth analysis (by microarray and Luminex®-based arrays) suggests a robust secretoma in favor of wound healing properties. Clinically speaking, iASC are good candidates for ischemic wound healing, even if clinical experience in humans remains very sparse in contrast to animal models [5]. Lee et al. published a small pilot study using ASC in 15 patients with critical limb ischemia. They injected 3 × 108 ASC/ml at different points in limb muscles and achieved improvement of ischemia in 67%, but there were only three patients with diabetic foot ulcers. The authors nevertheless demonstrated the feasibility of their therapeutic approach [12]. Other authors tried the same. Gadelkarim et al. performed a recent extensive literature review on ASC administered via scaffolds and/or skin grafts into chronic wounds [13]. In their review, authors injected different doses of ASC, ranging between 2 × 104 and 5 × 107 cells. Chronic ischemic wounds treated with ASC exhibited significantly higher healing rates. However, as a major drawback in the current literature, the majority of the wounds investigated were on other regions; and nota bene not in the distal diabetic foot [14,15]. Wound healing involves multiple steps including (i) inflammation, (ii) cell growth factor activity, (iii) angiogenesis (iv) tissue remodeling. Inflammation is first required to attract locally neutrophils for the phagocytosis of debris, pathogens and damaged tissue, in addition to releasing signaling molecules that initiate the proliferative phase of wound healing. One of the key elements for this step is macrophages that release potent pro-inflammatory cytokines such as IL-1, IL-6 and IL-8 (CXCL-8). Via production of these three cytokines, iASC could contribute to enhance the inflammatory step of healing. In accordance with those properties, iASC could enhance macrophage activity through CSF-1 (M-CSF). CSF-1 is a hematopoietic growth factor that is involved in the proliferation, differentiation, and survival of macrophages, also increasing their phagocytic and chemotactic activity. Stimulated on site by hypoxia, they produce angiogenic and cell growth factors to re-epithelialize the wound, create granulation tissue and lay down a new extracellular matrix. Neovascularization follows inflammation during the healing process. This process is concomitant with fibroblasts proliferation when endothelial cells migrate to the area of the wound during inflammation. Macrophages consequently switch from a pro-inflammatory to a healing mode through secretion of chemokines and VEGF. Chemokines attract adjacent endothelial cells that respond by forming capillaries and new vessels. To ensure this vascularization, endothelial cells need collagenases such as MMP to degrade fibrin and part of the extracellular matrix. iASC produces angiogenic factors such as VEGF, ANGPTL-2, ANGPTL-4, and adrenomedullin. VEGF is a potent angiogenic factor, whereas ANGPTL2 is a glycoprotein-promoting inflammation protein, serving in tissue reconstruction and angiogenesis [17-19]. ANGPTL4 harbors vasodilator properties through NO production and is also a potent angiogenic factor [16,17]. Tissue remodeling suited for angiogenesis is also suggested to be favored by iASC as the most abundant mRNA expression corresponded to Matrix-Metallo-Proteinases (MMP), with a predominance of MMP-1, 2, 3, 14, and 16. This not only suggests a major role of ASC in subcutaneous tissue remodeling or angiogenesis but equally reinforces their clinical interest.

Simultaneously with angiogenesis, fibroblasts must accumulate in the site to become the main cell in the wound. Again, macrophage and mast cells release FGFs and chemokines to activate fibroblasts from adjacent tissues, this process being possible through environmental MMPs [20,21]. The deposited fibroblastic connective tissue then forms granulation tissue prior to collagen production for a new matrix.

Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, and the components of a new extracellular matrix. FGFs, IFGs, PDGF, TGF-β, and fibronectin stimulate fibroblasts proliferation, migration to the wound, and production of the matrix. All of these factors are produced by iASC. The family of Insulin Growth Factor Binding Proteins (IGFBP) was the most represented in iASC, with notably the robust expression of IGF2BP2, IGFBP3, IGFBP4, IGFBP6 and IGFBP7 (Figure 4A). IGFBPs have several functions, including the promotion of Insulin-like Growth Factors (IGF) transport and activity, the latter harboring growth-promoting actions in many tissues of the body near the site of their formation, by the way of paracrine and autocrine effects. A profile of FGF expression shared between iASC was also observed, with the expression of FGF-2, FGF-5, and FGF-7 (Figure 4B) [20-22].

## Conclusion

In conclusion, ASC form ischemic patients show a secretome in favor of healing promotion. These regenerative properties are suggested to favor inflammation through the production of pro-inflammatory chemokines and factors enhancing macrophages activity. iASC also produces angiogenic factors, matrix remodeling proteases, chemokines, and keratinocytes/fibroblast growth inductors to help for later steps of healing. This pilot evaluation confirms the feasibility of harvesting, amplifying autologous ASC from superficial fat of ischemic patients ex vivo, as well as regenerative properties compatible with cell therapy of ischemic ulcers, including for the diabetic foot.

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## Compliance with Ethical Standards

This work has been financially supported by the Private Foundation of Geneva University Hospitals and Louis Jeantet Foundation. There are no conflicts of interests for any author. All patients participating in the study were informed and signed informed consent.

## Author Contribution

IU: conception and design, study conduct, study supervision, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript; MB: conception and design, collection and/or assembly of data, data analysis and interpretation; SM: provision of study material or patients, collection and/or assembly of data; MPH: collection and/or assembly of data; PBC: collection and/or assembly of data, data analysis and interpretation; PRL: collection and/or assembly of data, data analysis and interpretation; CM: collection and/or assembly of data, data analysis and interpretation; LKC: provision of study material or patients, collection of data; ER: provision of study material or patients, clinical work; DS: provision of study material or patients, clinical work; SI: data analysis and interpretation; NCB: financial support, collection and/or assembly of data; KHK: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript; OPS: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

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